

# Reflections

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### Discovery of DNA Polymerase

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DNA polymerase, an enzyme discovered in 1955, has the remarkable capacity to catalyze the template-directed synthesis of DNA (1, 2). The discovery of DNA polymerase has contributed in major ways to our present day understanding of how DNA is replicated and repaired and how it is transcribed. It also permitted the development of PCR and DNA sequencing, upon which much of modern biotechnology is based. In this article, I wish to recount how the discovery of the first DNA polymerase, DNA polymerase I of *Escherichia coli*, came about. I will say at the outset that although I am the author of this “Reflections” article, the discovery of DNA polymerase and the revelation that it is a template-directed enzyme resulted from the collective efforts of a small group that initially consisted of Maurice Bessman, Ernie Simms, and myself working with Arthur Kornberg in the Department of Microbiology at the Washington University School of Medicine. We were later joined by Julius Adler and Steve Zimmerman. The approach that we took reflected Arthur’s strong conviction that once a simple and quantitative assay could be established in a cellular extract, enzyme purification would reveal the enzymes involved and ultimately lead to the elucidation of the reaction(s) that they catalyzed. This approach, taken by Otto Warburg and the other great German biochemists in the 1920s and 1930s, had inspired Arthur Kornberg in the elucidation of coenzyme and nucleotide biosynthesis (3, 4). Bessman, Adler, and I were postdoctoral fellows, Zimmerman was a graduate student, and Simms was a technician.

#### ***Incorporation of [<sup>14</sup>C]Thymidine into Acid-insoluble, DNase-sensitive Product***

The story begins with Arthur’s discovery in December 1955 that [<sup>14</sup>C]thymidine, which he had obtained from Morris Friedkin in the Pharmacology Department at Washington University, could be converted in the presence of ATP to a cold trichloroacetic acid-insoluble product by an extract of log phase *E. coli*. The specific radioactivity of the <sup>14</sup>C-labeled thymidine was low, and fewer than 100 cpm above background were incorporated out of about 10<sup>6</sup> cpm added to the reaction. However, treatment of the product with crystalline pancreatic DNase, which had just then become available, rendered all of the radioactivity acid-soluble.

I had joined Arthur Kornberg’s laboratory in September of 1955 and had begun work on the purification of an enzyme in extracts of bacteriophage T2-infected cells that added a hydroxymethyl group to dCMP to form hydroxymethyl-dCMP. (In the T-even phages, cytosine is entirely replaced by hydroxymethylcytosine, which is present in various states of glucosylation (5, 6).) When Arthur showed me his results, I was tremendously excited by the possibility that they represented the first demonstration of DNA synthesis *in vitro* and I asked if I could put my project on hold and join him. He agreed. We learned later of Seymour Cohen’s discovery of dCMP hydroxymethylase, a discovery that opened up the whole field of virus-induced enzymes (7). I have never regretted my decision. Several months later Maurice Bessman arrived, and together with Ernie Simms, the four of us began to fractionate the activities responsible for the incorporation of the labeled thymidine into an acid-insoluble, DNase I-sensitive product.

Earlier that year, Arthur Kornberg and Ernie Simms had begun work on the purification of an activity in *E. coli* that converted thymidine in the presence of ATP to what was referred to as thymidine-X, later identified as dTMP. The activity was thymidine kinase. Additional products presumed to be dTDP and dTTP were also observed. The ability to make  $^{32}\text{P}$ -labeled dTMP was a significant step forward because we were not limited by the low radioactivity of the  $^{14}\text{C}$ thymidine that was available, and the  $^{32}\text{P}$ dTMP incorporated into the acid-insoluble product was now in the hundreds and occasionally in the thousands of counts/min. We bet that dTTP was the true substrate for our enzyme rather than dTMP or dTDP, although the latter was a distinct possibility because of the finding a year earlier by Grunberg-Manago and Ochoa that the nucleoside diphosphates rather than the triphosphates were the substrates for their ribonucleotide-polymerizing enzyme from *Azotobacter* (8), which later turned out to be polynucleotide phosphorylase, an enzyme involved in messenger RNA degradation. Once we had  $^{32}\text{P}$ dTMP, we prepared  $\alpha$ - $^{32}\text{P}$ dTTP by incubating our  $^{32}\text{P}$ -labeled dTMP with a partially purified nucleoside-diphosphate kinase and ATP and isolating the dTTP, which we carefully analyzed for thymidine, deoxyribose, and phosphate. These were present in the ratio of 1:1:3. Our assay mixture now consisted of a crude sonic extract of *E. coli*,  $\alpha$ - $^{32}\text{P}$ dTTP, ATP,  $\text{Mg}^{2+}$ , and buffer. As in the original experiment, acid-insoluble  $^{32}\text{P}$  was measured. With this assay, we began to fractionate the crude extract for dTTP incorporation into "DNA."

### **Purification of DNA Polymerase and Discovery of Deoxynucleoside Triphosphates**

To begin the fractionation we added streptomycin sulfate to the extract to produce a precipitate that contained the cellular nucleic acids and a nucleic acid-free supernatant. Streptomycin sulfate was used frequently at the time to remove nucleic acids, often a hindrance to protein purification in bacterial extracts. Assay of the nucleic acid-free supernatant (S-fraction) and the nucleic acid-containing precipitate (P-fraction) showed them to be devoid of dTTP incorporation activity. However, when the two fractions were combined, activity was restored. We also observed that prior incubation of the extract or the P-fraction for a few minutes at 37 °C substantially increased activity. Clearly more than one enzyme was required for the incorporation of dTTP into an acid-insoluble product. The complexity of the system became even more apparent when we began to fractionate S and P. The P-fraction could be subfractionated into two fractions, one heat-labile and the other heat-stable, both of which (in combination with the S-fraction) were necessary for activity. The S-fraction could be separated into a heat-labile fraction and a heat-stable fraction that could pass through a dialysis membrane, *i.e.* was dialyzable. The latter could be further fractionated by Dowex-1 chromatography into three discrete fractions. (Dowex-1, an anion exchange resin used at the time, separated low molecular weight acidic compounds.) Thus, incorporation of dTTP into an acid-insoluble product required: (i) two heat-labile fractions; (ii) a heat-stable fraction; (iii) three heat-stable, dialyzable, chromatographically distinct fractions; and (iv) ATP. In the absence of any one of these components, the activity was significantly diminished. Clearly, a lot was going on. The heat-labile component in the P-fraction turned out to be the enzyme that catalyzed phosphodiester bond synthesis and which we named DNA polymerase. The heat-stable, non-dialyzable component in the P-fraction was DNA. The heat-labile, non-dialyzable component of the S-fraction was a mixture of deoxynucleotide kinases, which together with nucleoside-diphosphate kinase produced the heat-stable, dialyzable mixture of dCTP, dATP, and dGTP (Fig. 1).

### **Multiple Functions of DNA in Reaction**

We reconciled these complex requirements for the incorporation of  $^{32}\text{P}$ -labeled dTTP into an acid-insoluble product as follows. The DNA in the extract and the P-fraction was degraded by endogenous nucleases to the deoxynucleoside monophosphates (dNMPs). (Recall that preincubation of the extract or the P-fraction significantly enhanced activity.) These were converted to the corresponding deoxynucleoside triphosphates (dNTPs) by the kinases in the S-fraction and ATP to generate dCTP, dATP, and dGTP. The heat-labile component of the P-fraction was the DNA polymerase. What about the DNA in the P-fraction? We speculated that the DNA served two functions in addition to being the source of the deoxynucleoside monophosphates. First, it protected the miniscule amount of the labeled DNA that was synthesized from degradation by the nucleases in the extract. Second, Arthur Kornberg had been strongly influenced by the work on glycogen phosphorylase in the Cori laboratory. In the case of glycogen phosphorylase, glycogen served as a "primer" for the addition of glucosyl units from

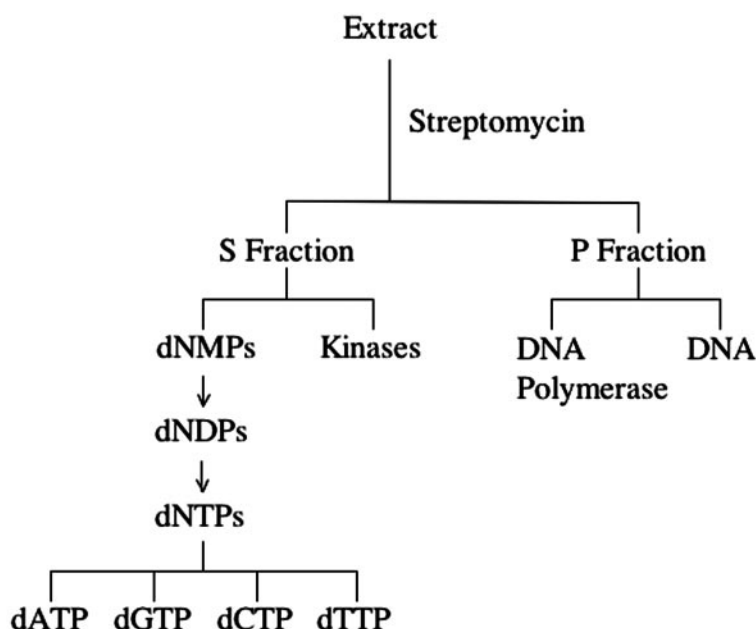


FIG. 1. Scheme for the separation of components required for synthesis of DNA by DNA polymerase.

glucose 1-phosphate to extend the glycogen chain (9). Similarly, he felt that dTMP from dTTP was being added to pre-existing DNA chains.

### Reconstruction of DNA Synthesis

Once the outlines of the reaction became clear, we set about the task of reconstituting the reaction with purified components. We partially purified each of the deoxynucleotide kinases in the S-fraction and with these kinases and nucleoside-diphosphate kinase synthesized and characterized the four dNTPs (dTTP, dCTP, dGTP, and dATP). This alone was a substantial advance, because with the exception of dTTP (10), none of the other dNTPs had previously been described.

To prepare the four  $^{32}\text{P}$ -labeled dNTPs we started with  $^{32}\text{P}$ -labeled DNA, isolated from  $^{32}\text{P}$ -labeled *E. coli*, from which we generated the four  $^{32}\text{P}$ -labeled dNMPs (dAMP, dCMP, dTMP, and dGMP) by treatment with pancreatic DNase and snake venom phosphodiesterase. The dNMPs were individually purified and converted enzymatically to the corresponding  $^{32}\text{P}$ -labeled dNTPs. Because the procedure usually consumed 2–3 weeks, the 14-day half-life of  $^{32}\text{P}$  necessitated the use of large quantities of  $^{32}\text{P}$  (50–100 mCi) in the 100-ml low phosphate culture medium that we used for the growth of *E. coli*.

### Isolation of DNA Polymerase

Purification of the DNA polymerase was a difficult and demanding task. The enzyme was present in relatively small amounts even in rapidly growing *E. coli* (about 300 molecules/cell). Fortunately, a fermentor for the large scale growth of *E. coli* that had been installed in the department supplied hundreds of grams of log phase *E. coli*. Later 100-pound batches of *E. coli* cell paste were obtained from the Grain Processing Corporation in Muscatine, IA. DEAE-cellulose and phosphocellulose, invented by Herbert Sober at the NIH, liberated us from the sole reliance on the ammonium sulfate, alumina  $\text{C}\gamma$ , and acetone fractionations that were the major protein fractionation tools at the time. With the aid of chromatography with these ion exchangers, we were able to obtain a several thousand-fold purified but not yet homogeneous preparation of the DNA polymerase. A vexing problem at the time was our inability to remove deoxyribonuclease activity from the enzyme. It was found later that there is a  $3' \rightarrow 5'$ -exonuclease activity associated with our *E. coli* DNA polymerase and indeed virtually all DNA polymerases, which serves a vital proofreading function, excising incorrectly incorporated nucleotides as replication proceeds (11).

### DNA Polymerase Takes Direction from a DNA Template

With the progress in fractionation the reaction requirements were now considerably simplified. Conversion of  $\alpha\text{-}[^{32}\text{P}]\text{dTTP}$  into an acid-insoluble product, *i.e.* DNA, required only the

partially purified DNA polymerase,  $Mg^{2+}$ , DNA, dCTP, dGTP, and dATP. We further found that *all four* dNTPs were absolutely required. With omission of any one of the other three dNTPs, incorporation of  $\alpha$ - $[^{32}P]$ dTTP fell to background levels. Here is how Kornberg put it in a symposium held in 1956 at Johns Hopkins University on "The Chemical Basis of Heredity": "In our current studies of DNA synthesis, we are dealing with a moderately purified protein fraction which appears to increase the size of a DNA chain. It does so only under the remarkable condition that all four of the deoxynucleoside triphosphates be present." (12).

The requirement for all four dNTPs was puzzling. If the DNA that we added was simply serving as a primer, why would all four dNTPs be needed? Was it possible that the DNA polymerase was performing the template-directed replication proposed by Watson and Crick for their double-stranded structure of DNA (13)? To test this idea we used DNAs with A+T/G+C ratios ranging from 0.5 to 1.9 as "primers." The result was stunning. The ratio in the product corresponded closely to that of the added DNA throughout the synthesis and was independent of the relative concentrations of the individual dNTPs. Even widely different relative concentrations of the dNTPs did not affect the base ratios of the DNA product. Clearly, the added DNA was serving as a template to direct the polymerase as it synthesized new DNA chains, or as we cautiously put it in our initial publication, "These results suggest that enzymatic synthesis of DNA by the "polymerase" of *E. coli* represents the replication of a DNA template." (2).

Having referred to the DNA added to the reaction as the "primer," by analogy to glycogen phosphorylase it was now clear that it also was serving as a template. However, we now know that all DNA polymerases require a primer to initiate a DNA chain. Various priming mechanisms (short RNA chains, proteins) have evolved to make up for this shortcoming in an otherwise magnificent enzyme (14–16).

### ***Initial Papers Rejected by the Journal of Biological Chemistry***

The two reports describing the preparation and characterization of the four dNTPs, the partial purification of the deoxynucleotide kinases required for their synthesis, the partial purification of the newly named "DNA polymerase," and the general properties of the polymerase reaction were declined by the *Journal of Biological Chemistry* when submitted in the Fall of 1957. Among the critical comments were: "It is very doubtful that the authors are entitled to speak of the enzymatic synthesis of DNA"; "Polymerase is a poor name"; "Perhaps as important as the elimination of certain banalities . . ." etc. Through the fortunate intervention of the late John Edsall, who had just assumed the position of Editor-in-Chief in May 1958, the two papers were accepted and appeared in the July 1958 issue (17, 18). Our paper which demonstrated that the polymerase copies the DNA template appeared in the *Proceedings of the National Academy of Sciences* in December of that year (2).

### ***Synthesis of a Copolymer of dAMP and dTMP "de Novo"***

By 1958, we had established that a DNA primer and template and all four dNTPs were required by our DNA polymerase for the synthesis of DNA. However, we received a rude shock one day when we observed, to our surprise, that with our most highly purified enzyme, DNA synthesis could proceed in the apparent absence of DNA. This episode is worth recounting in more detail. As I mentioned earlier, an important aim in purifying the DNA polymerase was to remove the contaminating DNase activity, which destroyed the product that we synthesized. Howard Schachman, the distinguished physical chemist, had come from Berkeley to spend a sabbatical year in the laboratory to analyze the DNA product of the polymerase reaction. Using the tools of the polymer chemist, the ultracentrifuge and the viscometer, Howard quickly demonstrated that the product of the reaction was indeed a large polymer. It then occurred to us that viscometry might be a very sensitive method to assay for nuclease activity in our most purified DNA polymerase preparations. I set up a nuclease reaction that contained calf thymus DNA,  $Mg^{2+}$ , and the DNA polymerase. At Howard's suggestion, dCTP, dTTP, and dATP were added to closely mimic the standard synthetic reaction conditions. dGTP (which was the most difficult of the triphosphates to prepare) was omitted to prevent DNA synthesis. The viscosity of the reaction mixture was then measured over an extended period. To my disappointment, the viscosity of the solution fell to that of the reaction buffer within about an hour. Obviously, the DNA was completely degraded; our best polymerase preparation was still contaminated with nuclease(s). This experiment was being performed on a Saturday morning at the same time that I was proctoring a microbiology exam for second year medical students



down the hall from the physical chemistry laboratory that Arthur Kornberg had set up for Howard. (All of us in the department, including postdoctoral fellows, participated in medical school teaching). After spending some time answering student questions and collecting exams, I returned to the laboratory to discard the reaction mixture and clean the viscometer. Before doing so, I absentmindedly took one last reading. To my amazement the viscosity of the solution had actually increased, and with repeated readings, the viscosity of the solution increased even further but then eventually fell back to that of the buffer. Could we be observing template-independent DNA synthesis? A number of control experiments were hastily performed that ruled out bacterial growth and contamination of the three dNTPs with dGTP. Howard Schachman and Julius Adler quickly found that the increase in viscosity required only the DNA polymerase,  $Mg^{2+}$ , dATP, and dTTP and occurred only after a lengthy lag. The product was a copolymer of alternating dAMP and dTMP, d(A-T) (19). Work several years later by Arthur Kornberg and Gobind Khorana showed that the rules had not been violated. The synthesis of the d(A-T) copolymer was indeed template-directed, but the template consisted of trace amounts of DNA present in the DNA polymerase preparation. The polymer was the result of a reiterative mode of DNA replication in which slippage of one stretch of alternating As and Ts within the contaminating DNA generated overlapping ends, which when filled in by the polymerase increased the chain length; ultimately, the high molecular weight d(A-T) copolymer was produced in quantity (20).

### ***Many Families of DNA Polymerases Have Been Discovered***

The DNA polymerase that we discovered is now called DNA polymerase I. In the intervening years, four additional DNA polymerases, DNA polymerase II, DNA polymerase III holoenzyme, and DNA polymerases IV and V, have been identified in *E. coli* and purified. The multisubunit DNA polymerase III holoenzyme is actually the enzyme that catalyzes the synthesis of the *E. coli* chromosome. DNA polymerase I, by virtue of its intrinsic ribonuclease H activity, together with its DNA polymerase activity plays an essential role in processing the nascent Okazaki fragments produced during the discontinuous replication of the lagging strand at the replication fork to prepare them for ligation. DNA polymerases II, IV, and V serve in the repair of DNA (reviewed in Ref. 21).

In eukaryotes the situation is even more complex. Fifteen distinct cellular DNA polymerases have been identified, and the list continues to grow (22). Three of these are devoted to replication of the genome (DNA polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$ ); DNA polymerase  $\gamma$  replicates the mitochondrial genome. The rest are all devoted to the repair of specific lesions in DNA. There are, in addition, virally encoded polymerases that replicate viral DNA genomes and in the case of the retroviruses reverse transcribe their RNA genomes into DNA. Despite the number and diversity of DNA polymerases, all of these enzymes show the same requirements that we observed nearly 50 years ago for the “polymerase” of *E. coli*: a template (DNA or RNA) to guide the polymerase in its base selection, a primer onto which deoxynucleotides are added, the four dNTPs, and  $Mg^{2+}$ . There are factors associated with DNA polymerases, *e.g.* clamps and clamp loaders, exonucleases, etc., which increase the efficiency and fidelity of DNA replication, but the basic mechanisms of replicating a DNA chain are all the same.

With the crystallization of many DNA polymerases, the determination of their three-dimensional structures (23), and the application of pre-steady state kinetic analyses (24), much is now known about the detailed chemical mechanism of the polymerase reaction. This information has been invaluable in the design of effective chemotherapeutic agents, in particular antiviral drugs.

### ***Envoi***

I view those days in the mid-1950s in the Department of Microbiology on the fourth floor of the old Clinic Building at Washington University to be among the most thrilling and enjoyable of my scientific career. There were new and unexpected findings being made virtually every day, and all of us in our small group shared in the joy and excitement of those discoveries. I feel terribly fortunate to have been part of that extraordinary moment in time.

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